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13. ABSTRACT (Maximum 200 Words)

T cell tolerance to tumor-associated antigens is a significant barrier to immune based treatments of human cancers. One such tumor-associated antigen is the proto-oncogene *HER-2/neu* (*neu*) which is overexpressed in 35-40% of all human breast cancers. Although patients with neu expressing tumors develop antibody and T cell responses to this antigen, these responses are weak and unable to hinder tumor growth. Our work has focused on understanding these mechanisms of T cell tolerance using the *neu*-N transgenic mice that express the wild type rat *neu* cDNA under control of the MMTV promoter. Since neu is an endogenously expressed antigen, profound neu-specific immune tolerance exists in the *neu*-N mice. We have characterized the immunodominant T cell epitope of neu recognized by parental FVB/N mice, $RNEU_{420-429}$. Studying T cell responses to this epitope has yielded important insights into the mechanisms of tolerance in the neu-N mice. Following a neu-targeted vaccine, 100% of FVB/N mice will activate T cells specific to RNEU420-429, whereas RNEU420-429-specific T cells are not activated in the neu-N mice. However, if vaccine is combined with immunomodulatory doses of chemotherapy in neu-N mice, RNEU420-420-specific are now activated in a subset of transgenic mice. Employing MHC tetramer technology, adoptive transfer of RNEU420-429-specific T cells, and T cell activation assays, we have begun to understand the mechanisms of tolerance that prevent the induction of protective immunity against tumors in the neu-N mice and ways to circumvent them. These findings are the basis for a Phase I Clinical Trial now underway at our institution.

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Introduction

In humans, the proto-oncogene *HER-2/neu* (*neu*) is overexpressed in 35-40% of all breast cancers (1). Although patients with neu expressing tumors develop antibody and T cell responses to this antigen, these responses are weak and unable to hinder tumor growth. This suggests that T cell tolerance is a significant barrier to immune based treatments that target neu and most likely other antigens expressed by breast cancers. *HER-2/neu* (*neu*-N) transgenic mice express the wild type rat *neu* cDNA under control of the MMTV promoter (2). Female mice spontaneously develop focal mammary tumors which overexpress the transgene in a stochastic manner starting approximately at 4 months of age. Since neu is an endogenously expressed antigen, it is likely that neu-specific immune tolerance exists in the *neu*-N mice. Three findings strongly support the existence of neu-specific tolerance in these mice. First, subcutaneous injection of 100-fold less transplantable *neu*-expressing mammary tumors is required for tumor growth in the *neu*-N mice when compared with the parental FVB/N mice. Second, neu-specific vaccines designed to prevent tumor development provide long-term prevention of *neu*-expressing tumors in the FVB/N mice but only delay slightly tumor growth in the *neu*-N mice. Third, neu-specific CD8$^+$ T cells (CTL) derived from FVB/N mice can lyse neu-expressing mammary tumors much more effectively than T cells derived from *neu*-N mice. (3) Based on these findings, the *neu*-N transgenic mouse model of mammary tumors provides a clinically relevant model for developing vaccines that overcome tumor tolerance. The overall aim of this work is to determine the mechanisms by which immune tolerance of CTL inhibits effective immunization in the *neu*-N transgenic mice and discover ways to circumvent them to increase the effectiveness of cancer vaccines.

Body

Identification of specific HER-2/neu MHC class I peptide epitopes

To study the mechanisms of CD8$^+$ T cell tolerance to neu in the *neu*-N mice, we first identified an immunodominant, MHC class I restricted T cell epitope from the neu protein. This research is described in detail in the attached manuscript in the appendix from the *Journal of Immunology* (*J. Immunology*, 2003, 170: 4273-4280). Briefly, neu-specific T cell lines were generated from vaccinated FVB/N mice by repeated *in vitro* stimulation with neu-expressing target cells. T cell lines, determined to be neu-specific by *in vitro* lysis assays, were then screened for activity against 9 cell lines, each expressing a fragment of the whole neu protein. Several T cell lines were activated by fragment 4 of neu. The T cell lines were then screened for activity against overlapping 10-mer peptides spanning the sequence of fragment 4. Each line reacted to the 10-mer comprising amino acids 420-429 (PDSLRDLSVF) of neu. We also determined that RNEU$_{420-429}$ is naturally processed and presented by tumor cells and is the optimal epitope in comparison to 8- and 9-mer derivatives.

We also generated T cell lines from *neu*-N mice given a neu targeted vaccine. Although multiple neu-specific lines were grown, only one recognized RNEU$_{420-429}$. Significant efforts have been made to identify other neu T cell epitopes recognized by the other T cell lines. The method described to identify the RNEU$_{420-429}$ epitope was unsuccessful. None of the *neu*-N-derived T cell lines recognized any of the 9 neu fragments. A combinatorial peptide library was also screened with the *neu*-N derived T cells employing a method others have used to discover T

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cell eptitopes (4). Again, after several attempts, no T cell epitopes could be determined from the data. We are continuing efforts to discover other neu T cell epitopes by utilizing bone-marrow derived dendritic cells that are infected with recombinant Listeria that express fragments of neu. We are also undertaking a large peptide synthesis approach where we are synthesizing overlapping 10-mers across the whole neu sequence.

Upon further study, we have shown by *ex vivo* analysis of T cell activation that all FVB/N mice given a neu-targetted vaccine generate $RNEU_{420-429}$-specific T cells, whereas $RNEU_{420-429}$-specific T cells are rarely observed in *neu*-N mice given the same vaccine. Hypothesizing that functional T cell tolerance to $RNEU_{420-429}$ in the *neu*-N mice could be one of the main mechanisms preventing effective anti-tumor immunity, we invested a significant amount of time and resources in developing reagents to study $RNEU_{420-429}$-specific T cell responses.

*Construction of H2-D^q RNEU$_{420-429}$ dimer and tetramer*

To study the T cell Receptor (TCR)/MHC class I/peptide interaction of $RNEU_{420-429}$-specific T cells, a MHC class I dimer loaded with $RNEU_{420-429}$ was made according to established protocols (5). Specific staining of $RNEU_{420-429}$-specific T cells was observed (data not shown), however the dimer reagent did not provide the sensitivity to detect T cell populations *ex vivo* nor could be used to estimate T cell receptor avidity. MHC tetramers have been shown to provide much cleaner staining of T cell lines and can detect T cells specific to the bound peptide epitope directly *ex vivo*. Tetramer has also been used to study the avidity of TCR/MHC/peptide interactions. Because of these issues, we chose to not use the MHC dimer, but instead we made a H2-D^q-RNEU$_{420-429}$ Tetramer. Specific staining of the $RNEU_{420-429}$-specific T cell lines (**Figure 1a**) was observed as well detection of $RNEU_{420-429}$-specific T cell populations *ex vivo* following a single *in vitro* stimulation (**Figure 1b**).


Figure 1.
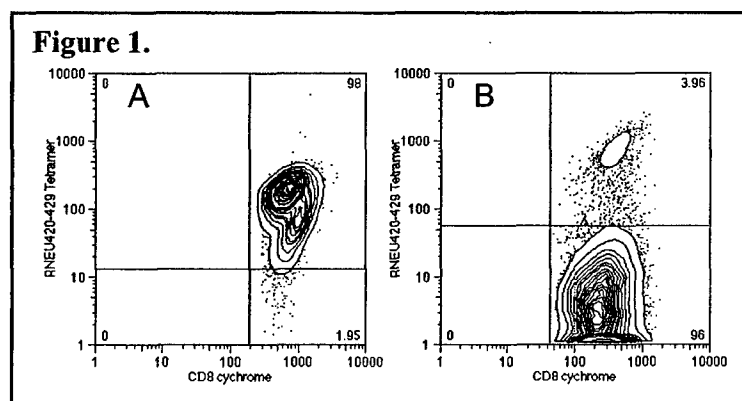
Analysis of T cell peptide epitopes for MHC class I binding affinity

Affinity for peptide epitopes for its cognate MHC class I molecule impacts how it is presented by antigen presenting cells (APC) and how well the TCR interacts with the APC. Several methods are available to test binding affinity. Surface plasmon resonance gives very precise measurements of binding affinities, yet is very labor intensive. As our intent is to simply determine which epitopes would be most effectively presented to T cells, we decided to use assays that give a more functional readout of peptide binding as opposed to a precise binding constant. We first determined the optimal peptide length of $RNEU_{420-429}$ for binding to MHC I. A T2 cell binding assay was performed on the peptides of interest. T2 cells lack the TAP transporter and as a result cannot present endogenous peptides on MHC I. The lack of peptide

causes the MHC to be unstable and be quickly internalized and degraded. Peptide binding can be assayed by incubating the T2 cells in serum free media containing the peptide of interest and then staining for MHC class I. Cells are then fixed and analyzed by flow cytometry. The level of MHC I expression is measured and will be highest on cells incubated with the peptide that binds to MHC I the best. We transfected T2 cells with murine H2-D^q to assay the binding of neu peptide epitopes. 8-mer and 9-mer variants covering $RNEU_{420-429}$ were assayed. **Figure 2** plots the mean fluorescence intensity of MHC I staining after the T2-D^q cells were incubated with different peptides. The 10-mer $RNEU_{420-429}$ was determined to be the optimal length for binding to H2-D^q.


Figure 2.
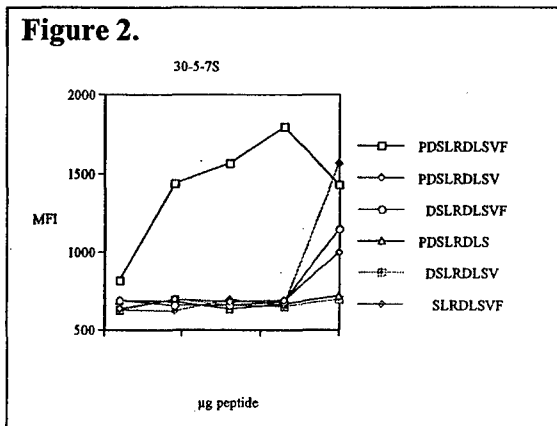
It was also noted that if the terminal phenylalanine at position 10 was removed no binding was observed, indicating its role as perhaps a crucial anchor residue for peptides in H2-D^q. To further investigate the role of each residue for MHC binding, alanine substitutions at each position were made and binding to T2-D^q cells was measured (**Figure 3**). When alanine was substituted at positions 4 and 10 of $RNEU_{420-429}$, binding to T2-D^q cells was abolished. These results indicate that the leucine at position 4 and the phenylalanine at position 10 are critical for binding to H2-D^q and serve as anchor residues for binding in the peptide binding groove of H2-D^q. The position of the anchor residues for H2-D^q has not previously been reported.


Figure 3.

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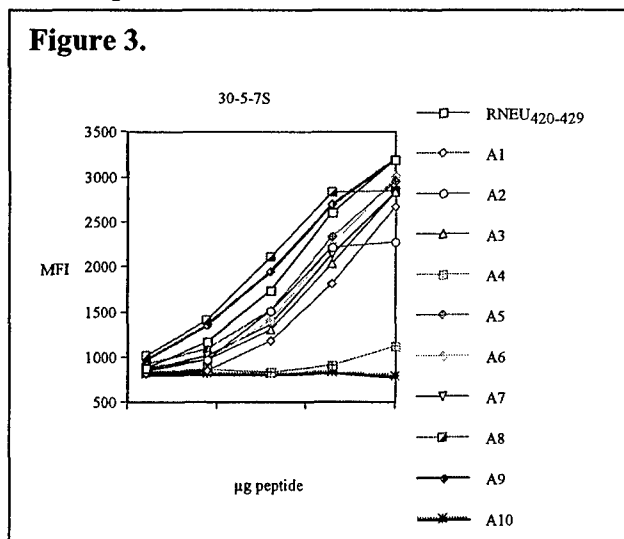
The second assay used to study peptide binding is an *in vitro* lysis assay employing T cells specific to the peptide epitopes being tested. Target cells, in our case T2-D^q cells, are pulsed with serial dilutions of each peptide and labeled with ^{51}Cr. The targets are then incubated with the T cells and, after 4 hours, lysis of the targets is measured by determining the level of ^{51}Cr in each sample. Peptides with higher binding affinities will cause increased lysis at lower concentrations. The results of these assays are included in the next section as we used the assay to determine variants of RNEU$_{420-429}$ that bind to H2-D^q with higher affinity.

Create amino acid substitutions in RNEU$_{420-429}$ to generate a more immunogenic peptide

Other systems have shown that T cell epitopes can be rendered more immunogenic by varying the peptide to bind more tightly to MHC I (6). As tolerance to RNEU$_{420-429}$ in *neu*-N mice appears to contribute to the lack of effective antitumor immunity, we searched for altered peptide analogues of the immunodominant neu peptide that had enhanced immunogenicity in our system. Altered forms of RNEU$_{420-429}$ were created by substituting alanine at each of the 10 positions. This type of approach has been successful in identifying heteroclitic T cell peptides in both rodent and human settings (6). In the majority of cases, substitutions did not enhance recognition. However, when alanine was substituted for glutamate at position 2, this peptide (designated RNEU$_{420-429}$A2) demonstrated markedly improved recognition by a FVB/N-derived T cell clone in the lysis assay described above as compared with wild-type peptide (**Figure 4**).

Figure 4.
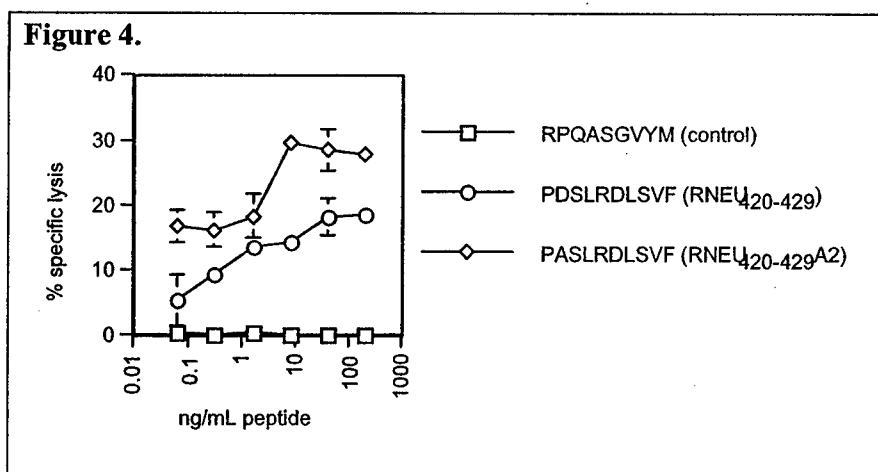
To determine if this heteroclitic peptide can immunize mice against mammary tumor expressing the natural RNEU$_{420-429}$ epitope, both wild-type RNEU$_{420-429}$ and the heteroclitic variant RNEU$_{420-429}$A2 were used to vaccinate mice. Dendritic cells derived from FVB/N mice were pulsed *in vitro* with either of these peptides (or with an irrelevant peptide) and then injected subcutaneously into FVB/N and *neu*–N mice followed by a subcutaneous challenge with the neu-expressing mammary tumor line NT2. As shown in **Figure 5**, mice immunized with dendritic cells pulsed with the wild-type peptide developed tumor at about the same rate as mice immunized with an irrelevant peptide (p<0.15 for FVB/N mice, p<0.39 for *neu*-N mice). However, FVB/N mice immunized with the heteroclitic peptide showed a lag in tumor growth as compared to FVB/N mice immunized with the irrelevant peptide (**A**, p<0.012). Although not statistically significant, *neu*-N mice demonstrated a promising trend toward protection when vaccinated with the heteroclitic peptide (**B**, p<0.21).

Figure 5.
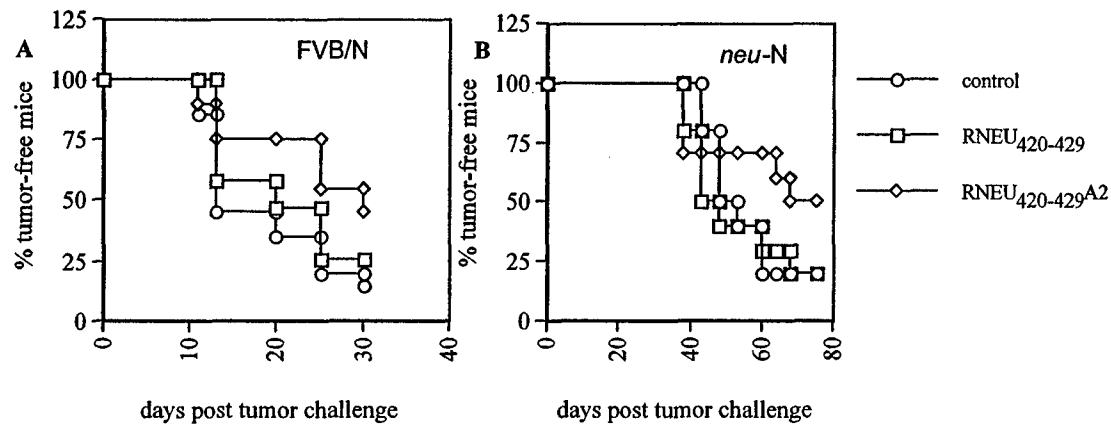
Determine avidity of RNEU$_{420-429}$-specific T cells for MHC/peptide complex

Several reports looking at TCR avidity to self antigens found that mechanisms of tolerance delete the CD8$^+$ T cells expressing the highest avidity TCR to self antigens, leaving only low avidity T cells that can be activated against a self antigen (7). If this is the case, tumor vaccines might only activate low avidity T cells specific for a tumor antigen that are poorly lytic and ineffective at eradicating tumor. Attempts to improve tumor vaccines could then focus on increasing the activity or quantity of these low avidity T cells. One such approach is vaccinating with a heteroclitic epitope as discussed above.

Another approach to improving vaccine efficacy that our lab has pioneered is combining vaccine with immunomodulatory doses of chemotherapy. Combining the granulocyte macrophage colony stimulating factor (GM-CSF)-secreting vaccine with cyclophosphamide (Cy) given 1 day before vaccine (100 mg/kg) and doxorubicin (Dox) given 7 days after vaccine (5 mg/kg) improves long term tumor free survival in *neu*-N mice to 20-30% as opposed to 0% long term survival in groups given vaccine alone or mock vaccine plus chemotherapy (8). Upon analysis of the T cell response generated in the vaccine + chemotherapy treated *neu*-N mice, RNEU$_{420-429}$-specific T cells were only found in mice that remained tumor free and never in mice where tumors grew out (data not shown). We wanted to test whether or not the T cells activated in the vaccine + chemotherapy treated mice were low avidity T cells that had been rendered more active by the chemotherapy or if the chemotherapy somehow allowed high avidity T cells for RNEU$_{420-429}$ to become activated.

Three RNEU$_{420-429}$-specific T cell lines were analyzed for TCR avidity using the H2-D^{q}RNEU$_{420-429}$ tetramer described above: 1. T cell line derived from vaccinated FVB/N mice 2. T cell line from *neu*-N mice given vaccine alone and 3. T cell line from *neu*-N mice that had remained tumor free following vaccine + chemotherapy. Each T cell line was shown to be uniformly reactive to RNEU$_{420-429}$ by intracellular cytokine staining following peptide stimulation (data not shown). The TCR and CD8 expression level of each line was also determined and shown to be equivalent (data not shown). Next each line was stained with increasing dilutions of tetramer (**Figure 6**). The cells were fixed and intensity of tetramer staining was analyzed by flow cytometry. If the T cell lines express equivalent levels of TCR, then tetramer staining intensity at each dilution will directly correlate with the strength of interaction between the TCR and the peptide/MHC complex. High avidity T cell lines will maintain higher tetramer staining levels at low tetramer concentrations, whereas a low avidity T

cell line will stain very poorly at low tetramer concentrations. The FVB/N line (**Figure 6a**) and the *neu*-N + chemotherapy line (**Figure 6c**) still showed a shift in tetramer staining at a 1:500 dilution, whereas the *neu*-N line (**Figure 6b**) showed no shift after the 1:5 dilution. From this data, it appears that the T cell line from the *neu*-N mice given chemotherapy is a high avidity T cell, despite being specific to an endogenous self antigen.

Figure 6.

Key

— 1/5 tetramer dilution
— 1/50 tetramer dilution
...... 1/500 tetramer dilution
.... unstained
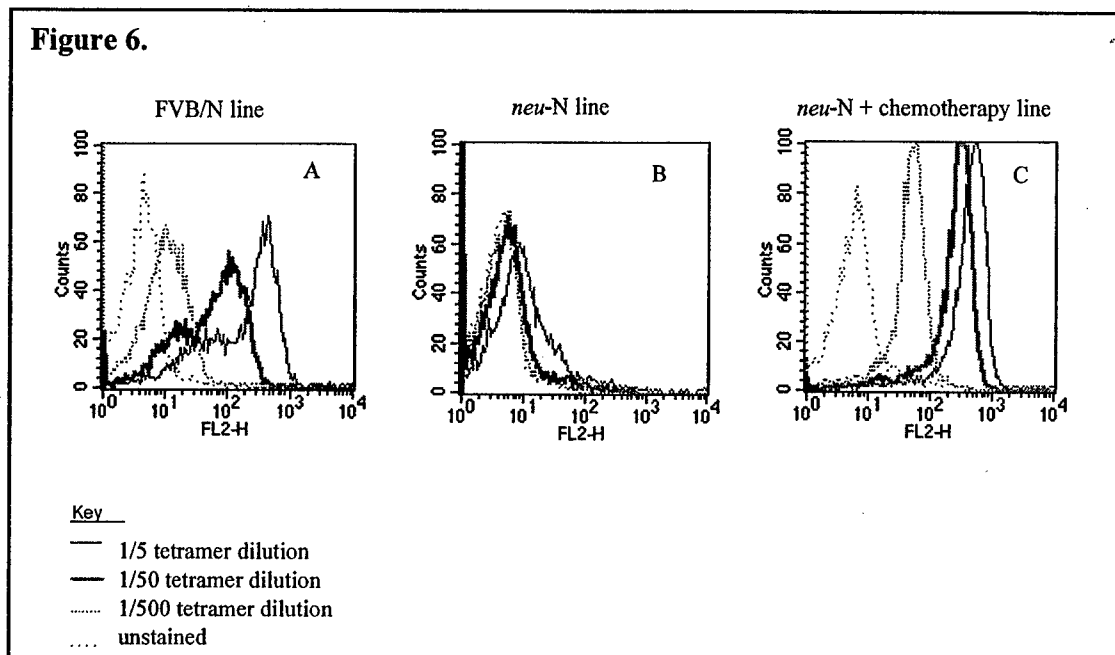
As this data contradicts findings in other models where only low avidity T cells to self antigens can be activated, we sought additional data to confirm these results. A "tetramer fall-off" assay was designed to determine the binding affinity of the TCR/MHC/peptide complex. Cells were first shown to have equivalent TCR expression levels. The lines were then stained with tetramer at the 1:5 dilution and excess tetramer was removed with two washes. Antibody to MHC I was then added in 20 molar excess and the samples were then assayed for tetramer staining level at time points up to four hours. Tetramer dissociates from the T cell according to the off-rate of the TCR/MHC/peptide complex and will then be bound up by the excess antibody to MHC I. The tetramer signal decreases over time according to the off-rate. These values were plotted and the off- rate was determined as the average of three independent experiments for each T cell line (**Table 1**). The low off-rates of the FVB/N and *neu*-N + chemotherapy lines compared to the *neu*-N line provide additional evidence that in the *neu*-N mice high avidity T cells to self antigen exist and be activated to the self antigen.

Table 1. k_{off} for $RNEU_{420-429}$-specific T Cell Lines

T cell line	k_{off} (min^{-1})	Stdev
FVB/N	41.5×10^{-3}	$+/-10.1 \times 10^{-3}$
***neu*-N**	272.7×10^{-3}	$+/-164.1 \times 10^{-3}$
***neu*-N + chemotherapy**	17.5×10^{-3}	$+/-3.9 \times 10^{-3}$

As the possibility exists that the results seen with the T cell lines are simply an artifact of long term culture, we next sought to confirm the data obtained from the T cell lines with experiments looking at the T cell response in the *neu*-N mice after only one *in vitro* peptide stimulation. *neu*-N mice were given a NT tumor challenge followed by vaccine + chemotherapy as described above. The spleens from *neu*-N mice that remained tumor free (6 of 42 mice), 3 tumor bearing *neu*-N mice, and 2 vaccinated FVB/N mice were harvested and stimulated for 7 days with $RNEU_{420-429}$. On day seven, $CD8^+$ T cells were enriched from the *in vitro* culture and stained for CD8, CD62L, and with increasing dilutions of tetramer. Cells were fixed and analyzed by flow cytometry. Events were gated on $CD8^+$, $CD62L^{lo}$ cells and tetramer staining intensity was compared between mice. **Figure 7** shows the plots from three representative mice. Despite mouse to mouse variability, when comparing $CD8^+$ T cells from tumor free *neu*-N mice given chemotherapy and FVB/N mice, both contained high avidity $RNEU_{420-429}$-specific T cells.


Figure 7.
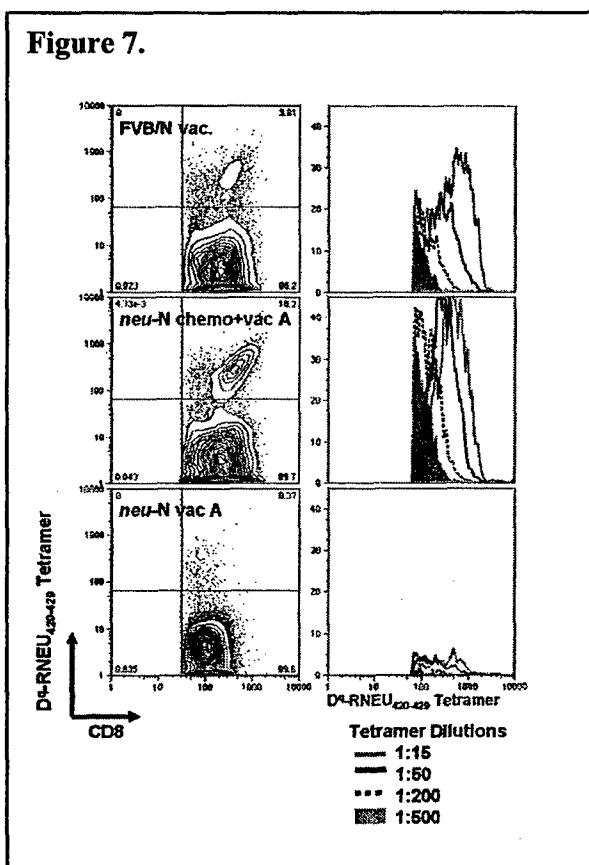
Our data from the *neu*-N mice suggest a model of tolerance where a spectrum of low avidity to high avidity T cells specific to self antigens (e.g. tumor antigens) exist in the periphery, yet if vaccine alone is given only the low avidity T cells are activated. However, if vaccine is combined with immunomodulatory treatments (e.g. the chemotherapy regimen we have described) the high avidity T cells can be activated and provide a potent antitumor effect. This represents a major paradigm shift in trying to improve the efficacy of tumor vaccines. As opposed to attempts to make low avidity T cells more active, understanding the mechanisms of tolerance that prevent the activation of the high avidity tumor-reactive T cells will yield even more effective vaccines. We have focused on adoptive transfer experiments both to track the fate

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of $RNEU_{420-429}$-specific T cells in the *neu*-N mice and to understand the mechanisms of tolerance that prevent the high avidity T cells from being activated.

Adoptive transfer of T cells from FVB/N and neu-N mice to evaluate their ability to cure pre-established mammary tumors

Having established the relative avidity for $RNEU_{420-429}$ of the three T cell lines from FVB/N, *neu*-N (referred to as low avidity *neu*-N), and *neu*-N + chemotherapy (referred to as high avidity *neu*-N), we performed adoptive transfer experiments to determine their effectiveness against neu expressing tumors *in vivo*. An influenza hemaglutinin-specific T cell line was used as a negative control (HA T cell line). The T cells were cultured and 1×10^7 cells were transferred into mice that had been challenged with tumor three days previously. Mice were monitored twice weekly for tumor growth. The mice were given twice daily injections until the completion of the experiment of interleukin 2 (IL-2). Four groups of mice were studied: 1. FVB/N (**Figure 8a**) 2. FVB/N given 100 mg/kg Cy the day before transfer (**Figure 8b**) 3. *neu*-N (**Figure 8c**) 4. *neu*-N mice given 100 mg/kg Cy the day before transfer (**Figure 8d**).

Figure 8
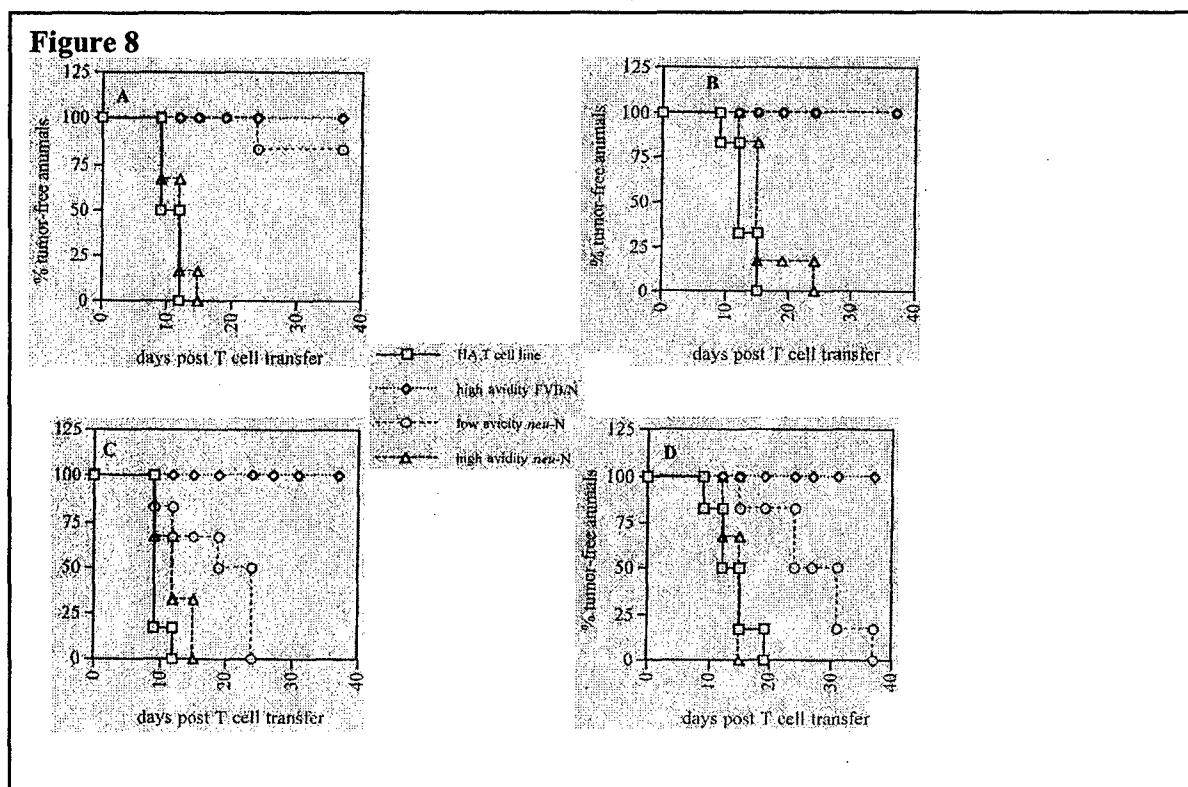
This *in vivo* data is surprising in part when compared to the *in vitro* data using the same cells. We hypothesized that the *neu*-N high avidity line would be more effective *in vivo* when compared to the *neu*-N low avidity line. However, the results showed the exact opposite effect. The low avidity line was effective in the FVB/N mice and delayed tumor growth the *neu*-N mice. The *neu*-N high avidity line did not even protect the FVB/N mice from tumor. We hypothesize that the differences of the *in vitro* to *in vivo* effect are due to the fact that they are T cell lines that have been grown in long-term culture. Differences in growth properties, sensitivity to growth factors, or other factors caused by culture could explain the differences. We are continuing to

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study the differences in these T cell lines by microarray analysis in the hopes of being able to better explain the differences in activity. Thus, as other confounding variables seem to be at play in the adoptive transfer experiments using the T cell lines, we have shifted the focus of our *in vivo* studies to using CD8$^+$ T cells directly from vaccinated mice that have not been cultured.

Tracking and functionality of adoptively transferred congenic T cells

We had initially hoped to be able to track transferred T cells using the H2-D^{q}RNEU$_{420-429}$ dimer or tetramer. This, however, proved to not be technically feasible. The dimer has very poor sensitivity and the tetramer is also not sensitive enough to track small populations without needing an *in vitro* peptide stimulation first. We also used Green Fluorescent Protein transduced T cell lines to track the T cells following transfer. Although the sensitivity was good enough to detect very few cells (data not shown), they could only be used for short term experiments as GFP is immunogenic and the mouse would mount an immune response against the transferred cells. The GFP T cells are also T cell lines and the problems discussed above with *in vivo* experiments would persist.

A marker used by many models is Thy1 (CD90). It is a membrane protein expressed at high levels on all T cells and two alleles exist (Thy1.1 and Thy1.2) that differ by only one amino acid. High quality antibodies are available that can distinguish between the two alleles. FVB/N mice express Thy1.1 so we performed backcrosses (10 generations) with Balb/c mice (expressing Thy1.2) to breed the Thy1.2 marker onto the FVB/N background. We can now use the FVB/N Thy1.2 mice as a source of T cells that can be easily tracked and analyzed after transfer into FVB/N or *neu*-N mice.

The first experiment was to determine the fate of high avidity T cells from FVB/N mice in *neu*-N mice. FVB/N Thy1.2 mice were vaccinated, generating high avidity RNEU$_{420-429}$ specific T cells. Two weeks after vaccine, CD8$^+$ T cells were purified from FVB/N Thy1.2 mice and 4x10^6 CD8$^+$ T cells were transferred into FVB/N or *neu*-N mice that had been vaccinated earlier in the day. Two weeks after transfer, spleens were harvested and the activity of CD8$^+$ T cells to RNEU$_{420-429}$ was assayed by intracellular cytokine staining for interferon gamma (IFN-γ). Cells were stained for Thy1.2 (Thy1.2$^+$ cells = transferred cells, Thy1.2$^-$ cells = host cells), CD8, and IFN-γ. **Figure 9** shows representative results from FVB/N mice (**Figure 9a**) and *neu*-N mice (**Figure 9b**) given vaccine and transfer of Thy1.2 T cells. The effector RNEU$_{420-429}$-specific Thy1.2 T cells proliferated and produced IFN-γ after transfer into the FVB/N host. However, the same T cells transferred into the *neu*-N mice were deleted, suppressed, or inactivated, as no IFN-γ-producing Thy1.2$^+$ T cells were observed. This finding suggests that mechanisms exist in the *neu*-N mice that do not simply prevent the activation of high avidity RNEU$_{420-429}$-specific T cells but can also inhibit the activity of previously activated T cells. We are currently investigating the cell type or factor that prevents the activation of these cells in the *neu*-N mice that is not present in the FVB/N mice.

Figure 9.

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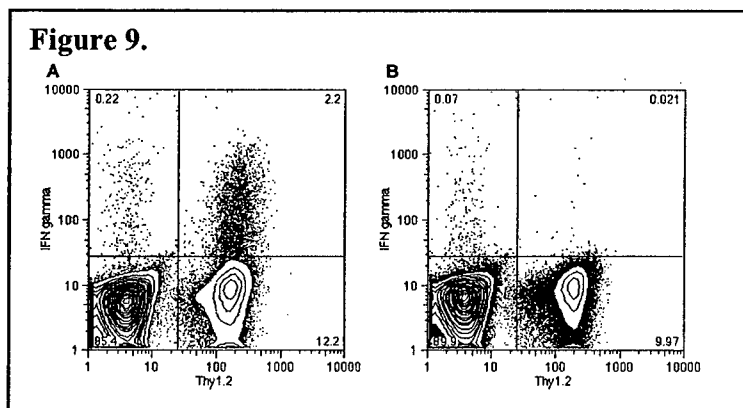
Characterizing cells that prevent the activation of high avidity RNEU$_{420-429}$-specific T cells
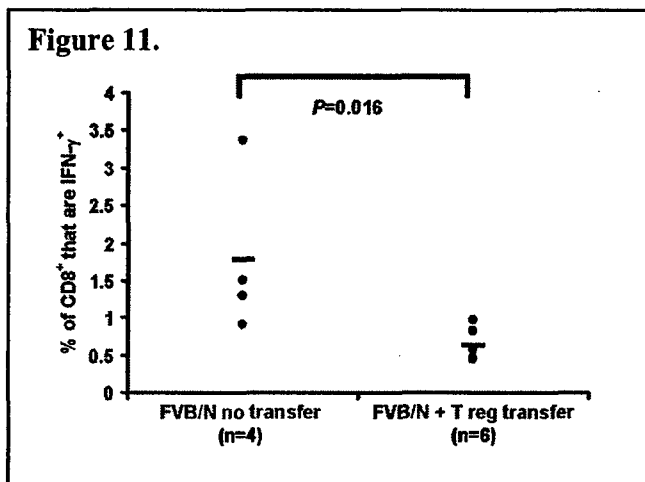
As we have shown that high avidity T cells to the neu self antigen can exist in *neu*-N mice, why these T cells are not activated by vaccine alone, but can be activated by vaccine + chemotherapy remains one of the most important questions. One hypothesis is that regulatory T cells (T$_{regs}$), which have been shown to play an important role in preventing autoimmunity (9), inhibit the activation of the high avidity T cells. A subset of CD4$^+$ T cells that express CD25 have been shown to act as T$_{regs}$ in several models (10). We hypothesized that this T cell subset might be suppressing the activation of the high avidity T cells and preventing effective antitumor immunity.

To test this hypothesis, *neu*-N mice were given vaccine + chemotherapy followed by tumor challenge two weeks after vaccine. One day prior to tumor challenge, groups of the *neu*-N mice received adoptively transferred T cell subsets from *neu*-N mice that received tumor and vaccine alone. If chemotherapy deleted or inhibited T$_{regs}$, then the adoptive transfer could restore the T$_{regs}$ and inhibit the antitumor response. If the T cell subset adoptively transferred did not contain T$_{regs}$, then tumor free mice would still be observed. **Figure 10** shows the results of transferring CD8$^+$ T cells (**Figure 10a**), CD4$^+$CD25$^-$ T cells (**Figure 10b**), or CD4$^+$CD25$^+$ T cells (**Figure 10c**). Only transferring the CD4$^+$CD25$^+$ subset resulted in suppressing the antitumor immune response.

Figure 10.
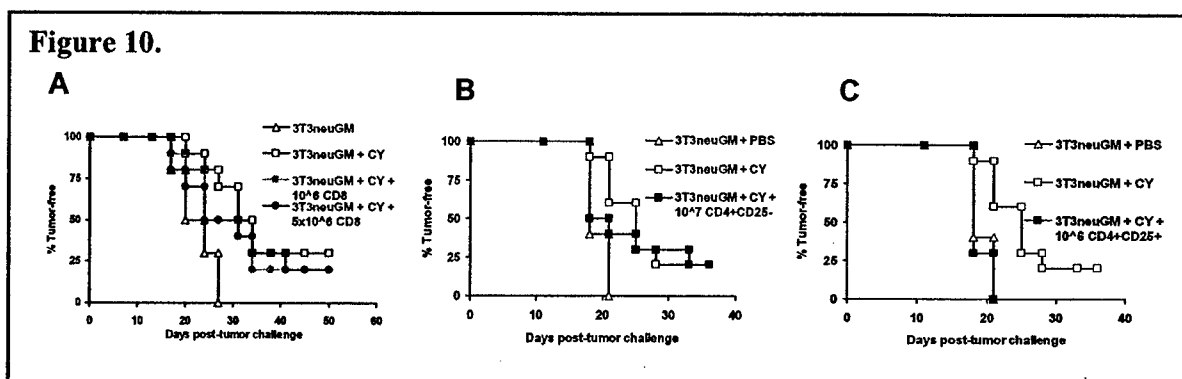
As CD4$^+$CD25$^+$ T cells in *neu*-N mice are able to inhibit the antitumor response in *neu*-N mice given vaccine + chemotherpay, we wanted to see if they could inhibit the immune response in FVB/N mice where no other mechanisms of tolerance to neu should be present. *neu*-N mice were given tumor challenge followed by vaccine. Two weeks later the CD4$^+$CD25$^+$ T cells were purified and 5x10^5 T$_{regs}$ were transferred into the FVB/N mice which were then vaccinated. Two weeks later the CD8$^+$ T cell response to RNEU$_{420-429}$ was determined in the FVB/N mice given transferred cells. As seen in **Figure 11**, the mice given CD4$^+$CD25$^+$ T cells from *neu*-N mice had significantly fewer (p=0.016) RNEU$_{420-429}$-specific T cells as determined by intracellular staining for IFN-γ. Although T$_{reg}$ transfer decreased the immune response in FVB/N mice, T$_{regs}$ did not completely inhibit the immune response to RNEU$_{420-429}$. As no response to RNEU$_{420-429}$ is seen in *neu*-N mice given vaccine alone, this indicates that although T$_{regs}$ must be playing a role in *neu*-N mice, other factors are also at play that prevent an effective antitumor immune response.

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Figure 11.

Plot: y-axis "% of CD8$^+$ that are IFN-γ$^+$" ranging from 0 to 4; bracket above groups labeled P=0.016; x-axis groups "FVB/N no transfer (n=4)" and "FVB/N + T reg transfer (n=6)".

Key Research Accomplishments

- Characterized the immunodominant MHC I restricted epitope of rat HER-2/neu in the FVB/N mice – RNEU$_{420-429}$.
- Determined the anchor residues for epitope binding to H2-D^q to be amino acids 4 and 10.
- Generated an altered, more immunogenic version of RNEU$_{420-429}$→RNEU$_{420-429}$A2.
- Demonstrated that vaccination of both parental and transgenic mice with the altered peptide resulted in improved tumor protection over vaccination with wild-type version.
- Generated the H2-D^{q}RNEU$_{420-429}$ Tetramer.
- By tetramer analysis, determined the relative avidity of the three RNEU$_{420-429}$-specific T cell lines – vaccinated FVB/N, vaccinated *neu*-N, and vaccine + chemotherapy *neu*-N.
- Determined that high avidity RNEU$_{420-429}$-specific T cells can be activated in *neu*-N mice if given vaccine + chemotherapy.
- Using adoptive transfer of congenic T cells, demonstrated that *neu*-N mice can actively suppress/inhibit high avidity FVB/N-derived RNEU$_{420-429}$-specific effector T cells.
- Characterized the CD4$^+$CD25$^+$ subset of T cells from *neu*-N mice as able to suppress the immune response in chemotherapy treated *neu*-N mice and non-tolerant FVB/N mice.

Reportable Outcomes

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- **A.M. Ercolini, B.H. Ladle**, E.A. Manning, L.W. Pfannenstiel, T.D. Armstrong, J-P.H. Machiels, J.G. Bieler, L.A. Emens, R.T. Reilly, and E.M. Jaffee. Recruitment of latent pools of high avidity CD8$^+$ T cells to the anti-tumor immune response. (manuscript in preparation)
- **Anne M. Ercolini**. Dissecting the mechanisms of CD8$^+$ T cell tolerance to the proto-oncogene *HER-2/neu* in the *HER-2/neu* transgenic mouse model of mammary cancer. Dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy. Baltimore, MD. February 2003.

- **B.H. Ladle, A.M. Ercolini**, E.A. Manning, L.W. Pfannenstiel, T.D. Armstrong, J-P.H. Machiels, J.G. Bieler, L.A. Emens, R.T. Reilly, and E.M. Jaffee. Recruitment of latent pools of high avidity $CD8^+$ T cells to the anti-tumor immune response. 12th Annual SPORE Investigator's Workshop – National Cancer Institute. July 10-13, 2004. Baltimore, MD. Abstract #226. Poster and Talk Presentation.
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- **Ercolini, A.M.**, Armstrong, T.D., **Ladle, B.H.**, Machiels, J.P., Lei, R., Reilly, R.T., and Jaffee, E.M. Presentation at New Investigators' Luncheon entitled "Dissecting the mechanisms of T cell tolerance for more effective breast cancer vaccine development." Department of Defense Breast Cancer Research Program Meeting, Orlando, FL, September 28, 2002. Abstract#P54-12.

Degrees/Employment
- Doctorate of Philosophy Degree in Immunology from The Johns Hopkins University School of Medicine awarded to **Anne M. Ercolini** in February 2003.
- Post-Doctoral Research Fellow – Position obtained by **Anne M. Ercolini** in March 2003 in the laboratory of Stephen D. Miller, Department of Microbiology-Immunology and Interdepartmental Immunobiology Center, Northwestern University Medical School, 303 E. Chicago Avenue, Chicago, IL 60611

Research Reagents
- GFP-expressing $RNEU_{420-429}$-specific T cell line
- $H2-D^q-RNEU_{420-429}$ MHC Tetramer
- FVB/N Thy1.2 congenic mice

Persons Receiving Salary Support
- Anne M. Ercolini, Ph.D. (July 2001 – March 2003)
- Brian H. Ladle, candidate for M.D./Ph.D. (March 2003 – July 2004)

Conclusions

Although the presence of antigen-specific CTL has been demonstrated in patients with tumor, the responses for the most part are weak and unable to hinder the growth of the malignancy. This has been shown in many types of cancer including neu-specific T cells in breast cancer. In some instances this may be due to an ineffective vaccine approach. However,

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in many cases mechanisms of peripheral T cell tolerance to specific tumor antigens may be at work. A major goal of cancer research is to develop therapies that will reverse the tolerant state and allow T cells to more effectively respond to tumor. *neu*-N transgenic mice display tolerance to the endogenous *neu* transgene and are therefore a clinically relevant model of breast cancer. The aim of our research has been to dissect the mechanisms of CD8$^+$ T cell tolerance in this mouse model. Knowledge of the mechanisms of tolerance in these mice will lead to the development of more effective vaccine strategies that can overcome tolerance to the neu antigen.

We identified RNEU$_{420-429}$ as the immunodominant epitope in rat neu, and that *neu*-N mice given vaccine alone develop low-avidity CTL specific for this peptide as compared with CTL from FVB/N mice. However, if vaccine is combined with immunomodulatory chemotherapy in *neu*-N mice, high avidity CTL specific for RNEU$_{420-429}$ are now generated. Importantly, the CTL are not only high avidity, but they are also able to eradicate tumor in the *neu*-N mice. Our research has focused on why these high avidity T cells can be activated only when vaccine is combined with chemotherapy and how the immune system prevents their activation when vaccine alone is given. We have shown that T$_{regs}$ are an important target of the chemotherapy and that they are able to suppress the antitumor response *neu*-N mice. We have also shown that other, as yet undetermined, mechanisms of tolerance also play a role in the *neu*-N mice.

Our work in the *neu*-N mouse model with combining vaccine and chemotherapy is the impetus behind a new clinical trial at our institution. Dr. Leisha Emens is currently enrolling patients on a trial combining a GM-CSF-secreting breast cancer vaccine with immunomodulatory doses of Cy and Dox. Along with clinical outcomes, blood samples are being taken to allow for the study of how the vaccine is working in humans. The findings of this trial will greatly enhance our understanding of immune tolerance to tumor antigens in humans and if chemotherapy is able to break tolerance similar to our observations in the *neu*-N mice.

Cancer vaccines alone have shown promise in treating cancer. However, great improvements in the efficacy of these vaccines are needed. Our work provides several critical insights into what approaches might be taken to improve the vaccines. First, we have shown that vaccine alone is not enough. Even though the vaccine is effective in a non-tolerant host, mechanisms of tolerance will prevent the activation of the most potent CTL in a tolerant host. Second, contrary to other reports, high avidity, tumor reactive T cells can persist in a tolerant host, yet they will not be activated if vaccine alone is given. And third, T$_{regs}$ represent promising targets that can be manipulated to allow the high avidity, tumor reactive CTL to be activated. Our future research will continue to focus on the mechanisms of tolerance in the *neu*-N mice with the goal of creating vaccine approaches that will activate the high avidity CTL that are most effective at eradicating tumor.

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Identification and Characterization of the Immunodominant Rat HER-2/neu MHC Class I Epitope Presented by Spontaneous Mammary Tumors from HER-2/neu-Transgenic Mice[1]

Anne M. Ercolini, Jean-Pascal H. Machiels,[2] Yi Cheng Chen, Jill E. Slansky,[3] Martin Giedlen,[4] R. Todd Reilly, and Elizabeth M. Jaffee[5]

The HER-2/neu (neu-N)-transgenic mice are a clinically relevant model of breast cancer. They are derived from the parental FVB/N mouse strain and are transgenic for the rat form of the proto-oncogene HER-2/neu (neu). In this study, we report the identification of a MHC class I peptide in the neu protein that is recognized by CD8^{+} T cells derived from vaccinated FVB/N mice. This 10-mer was recognized by all tumor-specific FVB/N T cells generated regardless of the TCR $V\beta$ region expressed by the T cell or the method of vaccination used, establishing it as the immunodominant MHC class I epitope in neu. T cells specific for this epitope were able to cure FVB/N mice of transplanted neu-expressing tumor cells, demonstrating that this is a naturally processed peptide. Altered peptide analogs of the epitope were analyzed for immunogenicity. Vaccination with dendritic cells pulsed with a heteroclitic peptide provided FVB/N and neu-N mice with increased protection against tumor challenge as compared with mice immunized with dendritic cells loaded with either wild-type or irrelevant peptide. Discovery of this epitope allows for better characterization of the CD8^{+} T cell responses in the neu-N mouse model in which neu-specific tolerance must be overcome to produce effective antitumor immunity. *The Journal of Immunology*, 2003, 170: 4273–4280.

An important role for Ag-specific CD8^{+} CTLs in antitumor immunity has been demonstrated in both murine tumor models and cancer patients. In mice, immunization with model and naturally expressed tumor Ags or their MHC class I (MHC-I)[6] epitopes induces antitumor CTL that mediate in vivo tumor rejection (1–12). Patients with established disease have shown some response when given adoptively transferred tumor-specific CTL (13–15). Similarly, clinical trials testing the efficacy of immunizing with MHC-I tumor epitopes from the human papilloma virus Ag E7 have resulted in T cell responses in some patients with cervical cancer (16–18). Several trials undertaken with peptides derived from melanoma-associated Ags have resulted in the induction and expansion of Ag-specific CTL. In some cases, immunization was also associated with clinical responses (19–22). However, in the majority of patients, immunization with MHC-I peptides induces T cell responses that are weak and ineffective in inducing significant tumor regression. As with all cancer therapies, the application of spontaneously arising mouse cancer models is central to the development of enhanced immunotherapies for human cancer (23).

We previously described neu-N-transgenic mice as a model of breast cancer that closely mimics immune tolerance described in some patients with cancer (24). These mice, derived from the parental FVB/N strain, express the wild-type rat HER-2/neu (neu) cDNA under the control of a mouse mammary tumor virus promoter (25). Female mice spontaneously and stochastically develop mammary tumors beginning at 4 mo of age. Mammary glands that have become tumorigenic overexpress the neu transgene relative to neu expression in normal glands in the same mice. Because the neu tumor Ag is endogenous to the host, this allows for the development of tolerance to the Ag, as evidenced by the poor ability of these mice to develop neu-specific antitumor immunity following vaccination as compared with the parental FVB/N mice. In the parental strain, depletion of CD8^{+} T cells before neu-specific vaccination inhibits their ability to reject a subsequent tumor challenge (24). Similarly, depletion of CD8^{+} T cells in transgenic mice before vaccination accelerates tumor outgrowth (26–29). However, unlike the parental mice, the transgenic mice are rarely cured of neu-expressing tumors. These data suggest that neu-specific CTL in neu-N mice are either weak lytic agents or are actively tolerized, either through deletion or anergy induction among high-avidity T cells.

The current study was designed to identify epitopes expressed by the rat neu and recognized by FVB/N (H-2^q)-derived CTL.

Identification and characterization of these epitopes are critical to understanding the difference in neu-reactive T cell responses between the transgenic and parental mice. Numerous tolerance studies have shown that T cells reactive with endogenous Ags expressed in the thymus are deleted (30–33), although some T cells can escape and exist in the periphery in a functionally unresponsive state (30, 34, 35). Similarly, T cells reactive with endogenous Ags not expressed in the thymus may be deleted or rendered nonresponsive in the periphery (36–38). Knowledge of the MHC-I epitopes in neu will allow us to study the fate of T cell responses directed at these epitopes in the neu-N-transgenic tolerant mice. In addition, it may be possible to alter these epitopes to identify heteroclitic epitopes with improved immunogenicity in the transgenic mice.

We have identified the immunodominant MHC-I epitope in the rat neu protein. This peptide is recognized by all neu-specific T cell lines and clones we derived from the splenocytes of vaccinated FVB/N mice, regardless of the TCR Vβ region expressed. In addition, we show that adoptive transfer of T cells specific for this peptide is protective in vivo. Furthermore, immunization with a heteroclitic version of this peptide is also protective against challenge with mammary tumors expressing the naturally processed epitope.

Materials and Methods

Peptides and primers

A panel of 135 peptides from fragment 4 (see Fig. 2) used in the initial screening were synthesized by Chiron (Emeryville, CA). All other peptides (>95% purity) were from the Johns Hopkins Biosynthesis and Sequence Facility (Department of Biochemistry, Johns Hopkins School of Medicine, Baltimore, MD). The PCR primers used to create the neu fragments (see Fig. 1) are as follows: fragment 1 (bp 1–508), 5'-CCGGGCCGAATTC GCAATGATC and 3'-CCCCGAATTCCTACTGAGGGTTCCCACGGA TCAA; fragment 2 (bp 458–886), 5'-GACATGAAGTTGCGGCTCC CTAGTCTCACAGAGATCCTGAAG and 3'-CCCCGAATTCCTACTC AGGGTTGTGCATGGACTC; fragment 3 (bp 836-1294), 5'-GACATGA AGTTGCGGCTCCCTGCCCTCGTCACCTACAACACA and 3'-CCCC GAATTCCTAGAGGTCACGGAGACTGTCTGG; fragment 4 (bp 1244– 1675), 5'-GACATGAAGTTGCGGCTCCCTATCACAGGTTACCTGTA CATC and 3'-CCCCGAATTCCTACTTCCATACTCGGCACTCCTC; fragment 5 (bp 1607-2077), 5'-GACATGAAGTTGCGGCTCCCTAC CCAGTGTGTCAACTGCAGT and 3'-CCCCGGTACCCTAGATCTTC TGTCTCCTTCGTTT; fragment 6 (bp 2009-2476), 5'-GACATGAAGT TGCGGCTCCCTGGCGTCCTGCTGTTCCTGATC and 3'-CCCCGGTA CCCTAACCTCGGTGTTCTCGGACATG; fragment 7 (bp 2405–2872), 5'-GACATGAAGTTGCGGCTCCCTTCCACAGTACAGCTGGTGACA and 3'-CCCCGGTACCCTAGCAGATTGGAGGCTGAGGTAG; fragment 8 (bp 2801–3271), 5'-GACATGAAGTTGCGGCTCCCTGATGG AATCCCAGCCCGGGAG and 3'-CCCCGGTACCCTACCCTTCC GAGGGAGCCAGTGG; and fragment 9 (bp 3203–3796), 5'-GACATGAAGTTGCGGCTCCCTGAGCTGACACTGGGCCTGGAG and 3'-CCCCGGTACCCTATACAGGTACATCCAGGCCTAG. Each fragment was ligated into the pcDNA3.1 mammalian transfection vector (Invitrogen, Carlsbad, CA) at the multicloning site.

Cell lines and medium

The IT22 cell line derives from a spontaneously transformed mouse fibroblast line as described previously (39). NIH 3T3 cells are a mouse fibroblast line (ATCC CRL-1658; American Type Culture Collection (ATCC), Manassas, VA). Rat neu cDNA was cloned from pSV2-neu-N (40) and ligated into the pcDNA3.1 vector (Invitrogen). IT22 cells were transfected with this construct by electroporation (20 μg/10^7 cells) to produce IT22neu cells. The human B7-1 gene was retrovirally inserted into rat neu-expressing NIH 3T3 cells (3T3neu; ATCC CRL-1915) using previously described methods (41) to produce 3T3neuB7-1 cells. The same neu-expressing NIH 3T3 cells were transduced with a murine GM-CSF retrovirus using the same methods for inserting the B7-1 gene to create 3T3neuGM vaccine cells. L-D^q cells and L-L^q cells are murine fibroblast cell lines transfected with murine H-2D^q or -L^q, respectively (42). All of the above cell lines were maintained at 37°C and 10% CO$_2$ in DMEM (Life Technologies, Rockville, MD) supplemented with 10% bovine calf serum (HyClone, Logan, UT), 1% L-glutamine (JRH Biosciences, Lenexa, KS), 1% nonessential amino acids (Sigma-Aldrich, St. Louis, MO), 1% sodium pyruvate (Sigma-Aldrich), and 0.5% penicillin/streptomycin (Sigma-Aldrich). The NT2 and

NT5B7-1 neu-expressing tumor lines are derived from spontaneous mammary tumors excised from the neu-N mice as previously described (24, 43).

Cloning and transfection of H-2D^q

Murine H-2D^q was cloned from the L-D^q cell line (5' primer, ATGGCTC CGCGCACGCTGCT; 3' primer, TCACGCTTTACAATCTCGGA) and ligated into pcDNA3.1. The T2 cell line is a B lymphoblast/T lymphoblast hybrid human cell line deficient in the MHC-I TAP transporter molecule (ATCC CRL-1992). These cells were transfected with D^q by electroporation (20 μg/1 × 10^7 cells) to create the T2D^q cell line. T2D^q cells were maintained at 37°C and 5% CO$_2$ in RPMI 1640 (Life Technologies) supplemented with 10% FBS (HyClone), 1% L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, and 0.5% penicillin/streptomycin.

neu-specific T cell lines and clones and hemagglutinin (HA)-specific T cell line

Some CD8$^+$ T cell lines were derived from FVB/N mice that were s.c. vaccinated with 1 × 10^6 irradiated 3T3neuGM cells at each of three sites (two forelimbs and one hindlimb). Mice were sacrificed, and spleens were excised 2 wk later. Splenocyte cultures were initially stimulated every 5 days with irradiated, IFN-γ-treated NT5B7-1 cells and then every 9 days by the addition of irradiated 3T3neuB7-1 cells as stimulators and FVB/N-derived splenocytes as feeders. Clones were developed from this line by limiting dilution. Other CD8$^+$ T cell lines were produced from the splenocytes of mice vaccinated with a neu-expressing recombinant vaccinia or neu plasmid DNA as described previously (24). A T cell line specific for the irrelevant Ag HA was derived from mice vaccinated with HA recombinant vaccinia virus as described previously (44). T cells were maintained at 37°C and 5% CO$_2$ in CTL medium (RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, 0.1% 2-ME (Sigma-Aldrich), and 0.5% penicillin/ streptomycin) supplemented with 10 cetus U/ml murine IL-2 (supernatant from B16 IL-2 line (45)).

Chromium release assays

Lysis assays were performed in triplicate in 96-well V-bottom plates as previously described (24). Briefly, target cells were resuspended in 100 μl of CTL medium and labeled with 0.2 mCi of ^{51}Cr/2 × 10^6 cells at 37°C and 5% CO$_2$ for 1 h. Cells were washed in CTL medium and resuspended at 6 × 10^4 cells/ml in RPMI 1640. To pulse peptide onto targets, 100 μl of peptide in RPMI 1640 was added to 50-μl targets for 1 h at room temperature in each well. After the removal of 100 μl of supernatant, 150 μl of T cells in CTL medium was added for the indicated E:T ratio. After a 4-h incubation, 100 μl of supernatant was assayed for ^{51}Cr release and percent specific lysis was determined by the formula: ((^{51}Cr release sample − spontaneous ^{51}Cr release target alone)/(maximum ^{51}Cr release target alone − spontaneous ^{51}Cr release target alone)) × 100. For the Ab blocking assays, the H-2D^q Ab 30-5-7S (ATCC HB-31) was added for 30 min at 37°C to 100 μl of target cells resuspended in CTL medium at a final concentration of 50 μM before addition of effector T cells.

Development and transfection of neu fragments

Nine overlapping fragments of the neu cDNA were created using specific primers and PCR amplification (Fig. 1A) and then ligated into pcDNA3.1. The fragment constructs were then transfected into NIH 3T3 cells using electroporation (20 μg/1 × 10^7 cells) or Lipofectamine (1.5 μg/3 × 10^5 cells; Life Technologies). Limiting dilution produced several clones of each fragment. RT-PCR was performed using primers described above to determine which clones expressed fragment mRNA.

Mice and dendritic cell immunizations

FVB/N mice were purchased at 6–8 wk of age from the National Cancer Institute (Bethesda, MD). neu-N mice (25) were bred to homozygosity as verified by Southern blot analysis. Splenic dendritic cells were generated from FVB/N mice as previously described (46). On the day of immunization, cells were collected, resuspended at 4 × 10^6/ml in AIM-V medium (Life Technologies), and pulsed with 300 μg/ml peptide for 3 h. Cells were then washed three times in HBSS (pH 7.4) and resuspended at 2 × 10^6 cells/ ml. Mice were given 0.1 ml s.c. injections in each hindlimb on days 0 and 7 and challenged with NT2 mammary tumor cells in the right hindlimb on day 14.

GM-CSF release ELISA

Peptides were pulsed onto NIH 3T3 cells (which were grown overnight at 26°C to increase the number of empty MHC on the cell surface) in RPMI 1640 at 26°C for 1 h at a final concentration of 0.1 μM. Targets were then

washed twice in CTL medium, and 1×10^5 T cells were added for a 1:1 E:T ratio. Plates were incubated at 37°C and 5% CO_2 for 24 h, and supernatants were harvested for the ELISA (Endogen, Woburn, MA). For the screening of *neu* fragments, 1×10^5 T cells and 3×10^5 targets were plated in CTL medium for 24 h and an ELISA was performed.

Flow cytometry (FACS)

Cells were stained by washing them two times in FACS buffer ($1\times$ HBSS (pH 7.4), 2% FBS, 1% HEPES (Life Technologies), and 0.1% NaN_3 (Sigma-Aldrich)) and incubating with Ab for 20 min at 4°C. Staining of TCR Vβ4, Vβ14, and Vβ17 was done using supernatants from hybridomas KT4-10, 14-2, and KJ23, respectively (47). Abs to Vβ2, -6, and -7, and fluorescence-conjugated secondary Abs were purchased from BD Phar-Mingen (La Jolla, CA). Ab to CD8 was purified from the 2.43 hybridoma (ATCC HB-27). MHC staining was done using supernatant collected from hybridomas 30-5-7S, 113, and 28-14-8S, which are specific for H-2D^q (42). The secondary Ab for all three Abs was FITC-conjugated goat anti-mouse IgG (BD PharMingen).

Intracellular cytokine staining (ICS)

ICS was performed as directed using a BD PharMingen kit for detection of murine IFN-γ. Briefly, 1×10^6 splenocytes (nylon wool purified to deplete B cells and macrophages) were incubated 12–16 h with an equal ratio of indicated targets in the presence of GolgiStop. Cells were washed in FACS buffer and stained with FITC-conjugated Ab to CD8, fixed and permeabilized, and stained with PE-conjugated Ab to IFN-γ.

Results

Generation of neu-specific CD8$^+$ T cells from vaccinated FVB/N mice

Three T cell lines were generated to characterize the T cell populations that result from different vaccination experiments as well as from different neu targeted vaccine approaches shown to be potent in other tumor models (48). A T cell line was therefore generated from mice vaccinated with the 3T3neuGM whole cell vaccine, and 11 neu-specific T cell clones were derived from this line. In addition, lines were created from mice vaccinated with the vaccinia vector expressing the entire neu protein and from mice vaccinated with neu plasmid DNA. All lines and clones were shown to lyse the full-length neu protein in a ^{51}Cr release assay using 3T3neu vs 3T3 wild-type targets (not shown). Analysis of the Vβ usage of these T cell lines and clones suggests that the neu CD8$^+$ T cell response is oligoclonal. Specifically, TCRs utilizing six Vβ regions (TCR Vβ2, -4, -6, -7, -14, and -17) were identified (data not shown).

Identification of RNEU$_{420–429}$, a peptide epitope contained in the extracellular domain of the rat neu protein, as the T cell target of all FVB/N-derived T cell lines and clones

As an initial approach to roughly map the position of neu epitopes, the *neu* cDNA was divided into nine approximately equal fragments overlapping by 15–25 aa (Fig. 1*A*). NIH 3T3 cells were
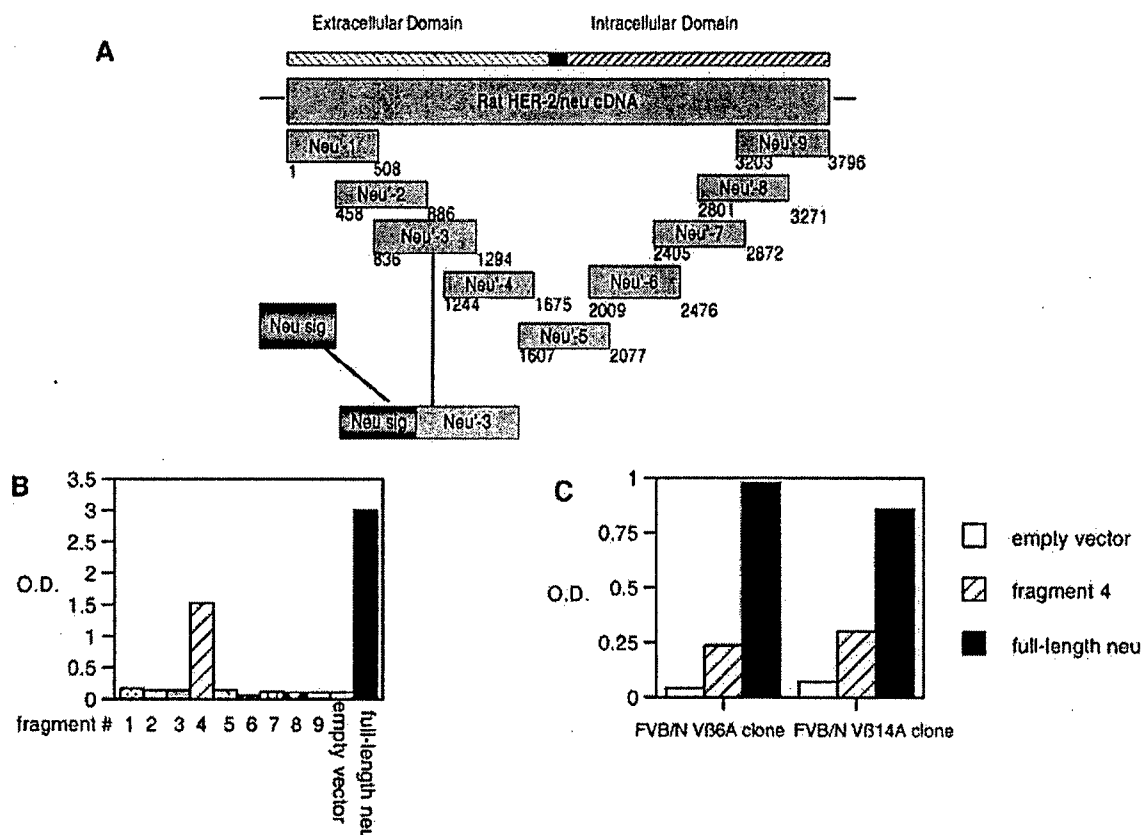
FIGURE 1. Extracellular fragment 4 of neu contains a neu MHC-I epitope recognized by the FVB/N-derived T cell clones. *A*, Nine overlapping fragments of *neu* cDNA were created using specific primers and PCR amplification. The numbers under each fragment refer to the starting and ending base pair within the entire *neu* cDNA. Each fragment was sequenced and confirmed to be the original rat *neu* sequence before ligation into the vector pcDNA3.1. The fragments were then transfected into 3T3 cells for use as targets in T cell assays. T cells (1×10^5) and 3×10^5 targets were plated in duplicate in 96-well plates and incubated at 37°C for 24 h. Supernatants were collected and tested for levels of GM-CSF by ELISA. Data are shown as degree of color change (OD). Effectors were FVB/N Vβ4A clone (TCR Vβ4) (*B*) and FVB/N Vβ6A clone (TCR Vβ6) and FVB/N Vβ14A clone (TCR Vβ14) (*C*). T cells show recognition of full-length neu (■) and of fragment 4 (▨).
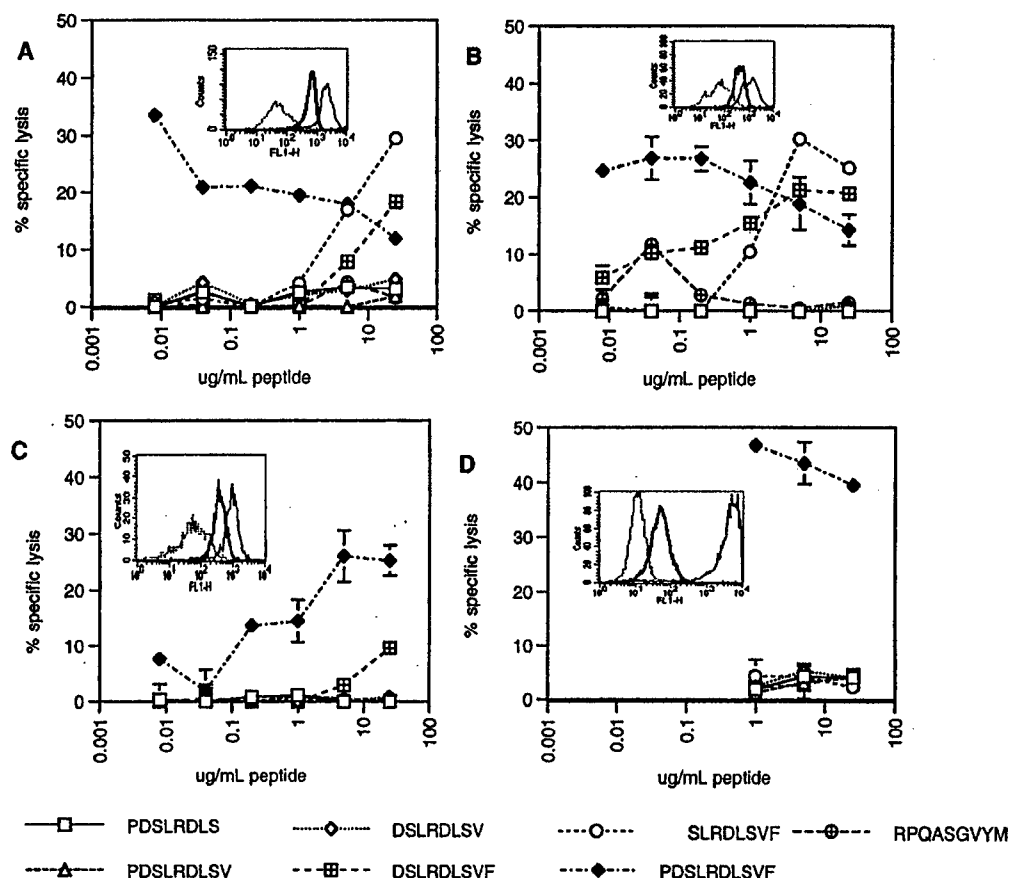
FIGURE 2. The original identified 10-mer RNEU$_{420-429}$ is the optimal epitope recognized by neu-specific T cell clones. Full-length and truncated versions of the epitope (as well as the irrelevant lymphocytic choriomeningitis virus nucleoprotein peptide NP$_{118-126}$ (68)) were pulsed onto 3×10^3 chromium-labeled IT22 cells for 1 h at 37°C. T cells were added before a 4-h incubation at 37°C. E:T ratios ranged from 15:1 to 25:1. Effectors were FVB/N Vβ2A clone (Vβ2) (*A*), FVB/N Vβ4A clone (Vβ4) (*B*), FVB/N Vβ6A clone (Vβ6) (*C*), and FVB/N Vβ14A clone (Vβ14) (*D*). *Insets*, Surface staining of T cell clones. Effectors were stained with Ab to CD8 (thin line), Ab to appropriate TCR Vβ region (thick line), or the irrelevant Ab rat IgG (dotted line).

transfected with these constructs and used as targets in GM-CSF release assays to identify which fragment contained the peptide(s) recognized by the clones. Fig. 1*B* shows recognition of 3T3 cells expressing fragment 4 (aa 410–553) by the FVB/N-derived T cell clone FVB/N Vβ4A (expressing TCR Vβ4). Recognition of fragment 4 was also seen with FVB/N Vβ6A clone (TCR Vβ6) and FVB/N Vβ14A clone (TCR Vβ14) (Fig. 1*C*). This fragment is located in the extracellular domain of neu near the transmembrane region.

Peptides 10 aa in length were synthesized, and these peptides spanned the entire sequence of fragment 4 (excluding the overlapping regions with fragments 3 and 5) and overlapped by nine residues each. Of the 135 peptides made, one (RNEU$_{420-429}$) was consistently recognized by clones in GM-CSF release ELISA (data not shown). This peptide (PDSLRDLSVF), along with its 8- and 9-aa derivatives, was synthesized and tested for recognition by neu-specific T cells in Cr^{51} release lysis assays. As shown in Fig. 2, the 10-mer was recognized more efficiently than the shorter peptides regardless of the TCR Vβ region expressed by the T cell. This peptide was subsequently found to be recognized by all T cell lines and clones derived, suggesting that RNEU$_{420-429}$ is the immunodominant MHC-I neu epitope recognized by FVB/N-derived T cells. To further analyze the CD8$^+$ T cell response to neu in FVB/N mice, frequency analysis was performed on the spleens of five individual mice given a s.c. 3T3neuGM vaccine. Fourteen days after vaccination, spleens were excised and cultured with 3T3neuB7-1 cells for 7 days before performing ICS to determine the response to full-length neu and to RNEU$_{420-429}$. As shown in

Table I, all vaccinated FVB/N mice developed CD8$^+$ T cells that were specific for both full-length neu and for RNEU$_{420-429}$. This further supports the immunodominance of RNEU$_{420-429}$ in

Table I. *T cell specificity of vaccinated FVB/N and neu-N mice[a]*

Mouse No.	% neu Specific[b]	% RNEU$_{420-429}$ Specific[c]
FVB/N 1	1.83	2.48
FVB/N 2	0.88	1.92
FVB/N 3	1.47	2.75
FVB/N 4	0.06	0.48
FVB/N 5	0.28	1.16
neu-N 1	0.00	0.32
neu-N 2	0.13	0.22
neu-N 3	0.00	0.23
neu-N 4	0.00	0.00
neu-N 5	0.02	0.26
neu-N 6	0.00	0.07
neu-N 7	0.00	0.06
neu-N 8	0.16	0.00
neu-N 9	0.00	0.04
neu-N 10	0.14	0.00

[a] Specificity determined by the percentage of CD8$^+$ splenocytes staining positive for IFN-γ in response to target in ICS assay.

[b] neu specificity determined by the percentage responding to IT22neu minus the percentage responding to IT22.

[c] RNEU$_{420-429}$ specificity determined by the percentage responding to T2D^q cells pulsed with RNEU$_{420-429}$ − the percentage responding to T2D^q cells pulsed with the irrelevant peptide NP$_{118-126}$.
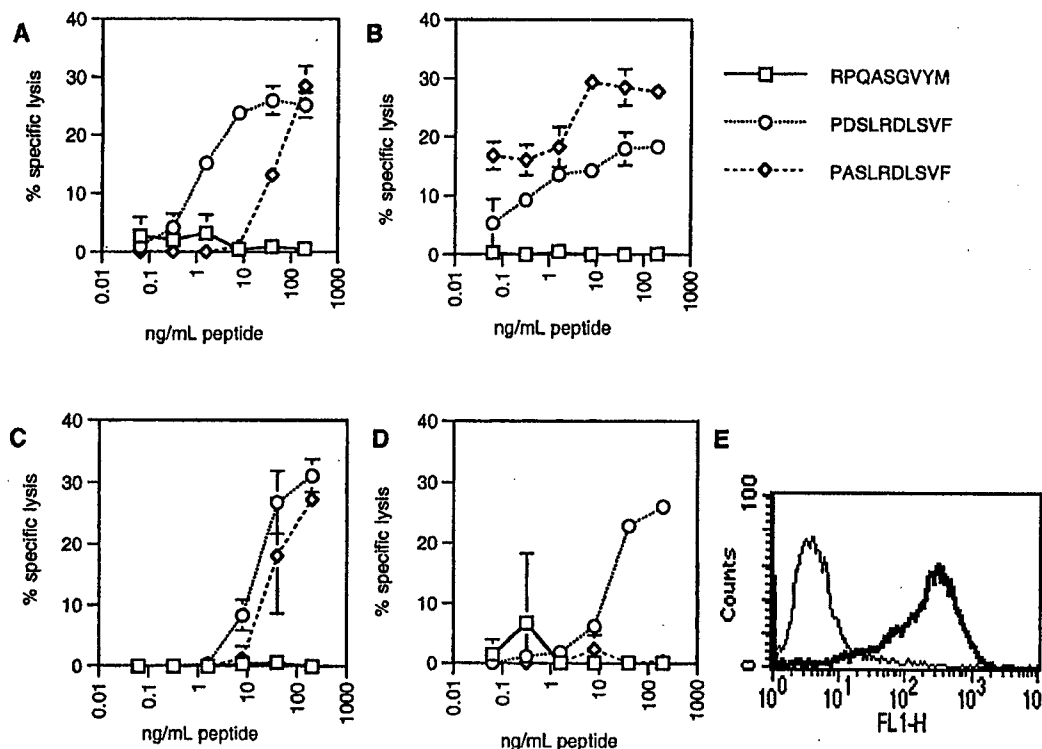
FIGURE 3. H-2D^q is the restricting MHC molecule for the recognition of RNEU$_{420-429}$ by FVB/N T cell clones. T2 cells were transfected via electroporation (20 μg/10^7 cells) with D^q cDNA to create T2D^q cells. Chromium-labeled T2D^q cells (3 × 10^3) were pulsed for 1 h with either RNEU$_{420-429}$ or the heteroclitic variant RNEU$_{420-429}$A2. T cells (3 × 10^4) were then incubated with the targets for 4 h at 37°C. Effectors were FVB/N Vβ2A clone (Vβ2) (*A*), FVB/N Vβ4A clone (Vβ4) (*B*), FVB/N Vβ6A clone (Vβ6) (*C*), and FVB/N Vβ14A clone (Vβ14) (*D*). T cell clone FVB/N Vβ4A demonstrates enhanced lysis of the RNEU$_{420-429}$ peptide with an alanine substituted at position 2 (RNEU$_{420-429}$A2). *E*, T2D^q cells were incubated with Ab to D^q (28-14-8S) followed by FITC-conjugated Ab to mouse IgG (thick line) to confirm surface expression of the MHC molecule (thin line, secondary Ab alone).

FVB/N mice. The same analysis was done on the spleens of 10 individual *neu*-N mice given the same vaccine. As shown in Table I, the tolerized *neu*-N mice do not demonstrate appreciable T cell activity for the immunodominant epitope.

H-2D^q is the restriction element for RNEU$_{420-429}$

Initial CTL blocking studies using an Ab specific for H-2D^q and -L^q (30-5-7S) indicated that lysis of neu was restricted to one of these two molecules. MHC restriction was further determined by pulsing RNEU$_{420-429}$ onto mouse L cells that were transfected with either H-2D^q or -L^q. FVB/N Vβ4A clone cells lysed targets transfected with D^q but not those transfected with L^q (data not shown). The D^q molecule was then cloned and transfected into T2 cells. When pulsed with RNEU$_{420-429}$, T2D^q cells were recognized in a ^{51}Cr release assay by all FVB/N-derived T cell lines and clones. Lysis of T2D^q cells pulsed with decreasing concentrations of the RNEU$_{420-429}$ peptide is shown in Fig. 3 for the four T cell clones also evaluated in Fig. 2. These data established D^q as the restricting MHC-I molecule for RNEU$_{420-429}$.

Adoptive transfer of RNEU$_{420-429}$-specific T cells into tumor-bearing FVB/N mice confirms that this peptide is a tumor rejection epitope naturally expressed by mammary tumor cells in vivo

FVB/N mice were given a s.c. leg injection of neu-expressing tumor cells (NT2) followed 1 day later by i.v. transfer of an FVB/N T cell line (derived from mice given neu plasmid vaccine) specific for RNEU$_{420-429}$. This line was chosen because it demonstrated the highest lysis of NT2 cells in vitro. As shown in Fig. 4, mice receiving these T cells showed protection from tumor outgrowth as

compared with mice receiving a control T cell line specific for the irrelevant Ag HA ($p < 0.0008$). This experiment was repeated using several RNEU$_{420-429}$ specific T cell clones and lines with similar results.
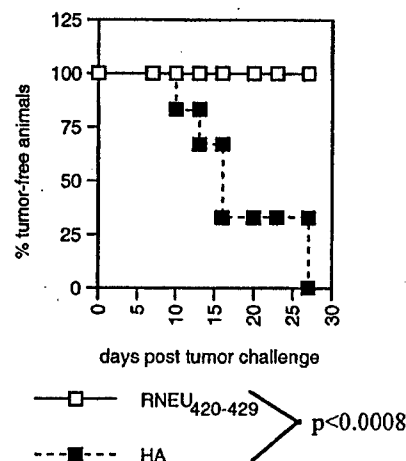
FIGURE 4. Confirmation that RNEU$_{420-429}$ is the naturally processed mammary tumor epitope expressed in vivo. FVB/N mice (9–10 mice/group) were given a s.c. leg injection of 5 × 10^6 neu-expressing tumor cells (NT2 cells derived from spontaneously arising mammary tumors in *neu*-N mice). One day later, mice were given an i.v. transfer of 1 × 10^7 CD8$^+$ T cells specific for either RNEU$_{420-429}$ or for the irrelevant Ag HA. Mice were observed twice a week for the development of tumor in the leg. Additional independent studies were performed with other T cell clones and lines with similar results.
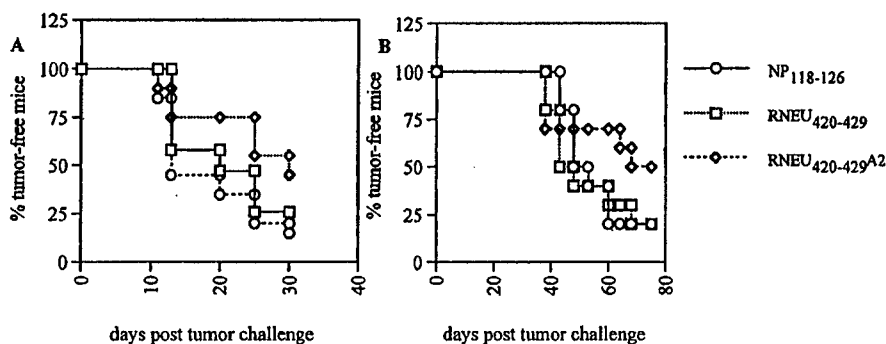
FIGURE 5. Mice immunized with the alanine substituted variant of $RNEU_{420-429}$ show a lag in neu-expressing tumor growth as compared with immunization with wild-type $RNEU_{420-429}$ or the irrelevant peptide. Splenic dendritic cells generated from FVB/N mice were pulsed with 300 $\mu g/ml$ either $RNEU_{420-429}$, $RNEU_{420-429}A2$, or the lymphocytic choriomeningitis virus $NP_{118-126}$ peptide. Subcutaneous injections (0.1 ml) of 2 × 10^5 cells were given in each hindlimb on days 0 and 7. Mice were challenged s.c. with NT2 tumor cells on day 14. *A*, FVB/N mice (19–20/group) were given 5 × 10^6 NT2 cells. *B*, neu-N mice (10/group) were given 5 × 10^4 NT2 cells. Mice were followed twice per week for development of leg tumor.

Identification of a heteroclitic peptide of RNEU$_{420-429}$ that can immunize mice in vivo against outgrowth by the mammary tumor

Identification of the immunodominant neu peptide allowed us to search for altered peptide analogs with potentially enhanced immunogenicity. Altered forms of $RNEU_{420-429}$ were created by substituting alanine at each of the 10 positions. This type of approach has been successful in identifying heteroclitic T cell peptides in both rodent and human settings (6, 49–54). In the majority of cases, substitutions did not enhance recognition. However, when alanine was substituted for glutamate at position 2, this peptide (designated $RNEU_{420-429}A2$) demonstrated markedly improved recognition by the T cell clone FVB/N Vβ4A in a lysis assay as compared with wild-type peptide (Fig. 3). Interestingly, the heteroclitic peptide was found to have a lower binding affinity than the wild-type $RNEU_{420-429}$ peptide in a T2D^q MHC stabilization assay (data not shown), suggesting that its improved stimulatory capacity was instead due to the enhanced stability of the TCR/MHC/peptide complex.

To determine whether this heteroclitic peptide can immunize mice against mammary tumor expressing the natural $RNEU_{420-429}$ epitope, both wild-type $RNEU_{420-429}$ and the heteroclitic variant $RNEU_{420-429}A2$ were used to vaccinate mice. Dendritic cells derived from FVB/N mice were pulsed in vitro with either of these peptides (or with an irrelevant peptide) and then injected s.c. into FVB/N and neu-N mice followed by a s.c. NT2 tumor challenge. As shown in Fig. 5, mice immunized with dendritic cells pulsed with the wild-type peptide developed tumor at about the same rate as mice immunized with an irrelevant peptide ($p < 0.15$ for FVB/N mice; $p < 0.39$ for neu-N mice). However, FVB/N mice immunized with the heteroclitic peptide showed a lag in tumor growth as compared with FVB/N mice immunized with the irrelevant peptide ($p < 0.012$). Although not statistically significant, neu-N mice demonstrated a promising trend toward protection when vaccinated with the heteroclitic peptide ($p < 0.21$).

Discussion

We have identified a MHC-I epitope in rat HER-2/neu, $RNEU_{420-429}$, which is the dominant target of the CD8$^+$ T cell response in FVB/N mice. This peptide, which is contained in the extracellular region of neu, is recognized by all neu-specific CD8$^+$ T cell lines and clones derived from vaccinated FVB/N mice regardless of the neu-targeted vaccine approach used for immunization. This was true even though vaccination induced an oligoclonal neu-specific T cell response as determined by the panel of

T cell lines and clones tested expressing several different TCR Vβ types. This was unexpected because the neu gene is 4kB in size and encodes for a large protein. However, other studies have reported similar findings demonstrating that a single viral or tumor antigenic epitope is recognized by a panel of T cells derived from immunized mice (8, 55–57). In contrast, neu-specific T cells isolated from patients with neu-expressing breast and ovarian cancer typically recognize multiple epitopes in both the extracellular and intracellular domains of the protein (58–63). Therefore, our data suggest that the FVB/N nontolerized mice recognize neu as a foreign Ag.

FVB/N mice were protected against inoculation with a neu-expressing in vitro tumor line (derived from naturally arising mammary tumors in neu-N mice) when T cells specific for this epitope were adoptively transferred. This indicates that $RNEU_{420-429}$ is the immunodominant MHC-I epitope in rat neu that serves as the naturally expressed tumor rejection target on spontaneously arising neu-expressing tumors. It is interesting to note that $RNEU_{420-429}$ differs from the corresponding murine amino acid sequence by 3 aa (64). Furthermore, $RNEU_{420-429}$ specific T cells do not recognize the murine peptide in a ^{51}Cr release lysis assay (data not shown). This probably explains its high degree of immunogenicity in the FVB/N mice.

Vaccination with the $RNEU_{420-429}$ peptide pulsed onto dendritic cells did not demonstrate antitumor immunity. This is not surprising, because vaccinating mice or patients with solely MHC-I tumor epitopes has produced only modest results in most studies. For some Ags, altering the wild-type MHC-I epitope so that it binds more strongly to MHC and/or demonstrates greater recognition in vitro by Ag-specific T cells improves the immunization potential and the clinical outcome (54, 65–67). In this study, we show that vaccinating FVB/N mice with dendritic cells pulsed with a heteroclitic variant of the wild-type epitope also induces improved protection against tumors that express the natural $RNEU_{420-429}$ epitope as compared with immunization with the $RNEU_{420-429}$ epitope itself. neu-N mice show a promising trend toward protection when vaccinated with the heteroclitic peptide but not to the degree seen in FVB/N mice. This may reflect the neu-specific tolerance seen in neu-N mice. In any case, this regimen proved to be far less efficacious for either strain of mice than our whole-cell vaccine, further supporting the concept that vaccines that induce both CTL and Th cell responses may be more effective than vaccines that only enhance the CTL response (41, 48).

Few studies have been published dissecting the immune responses in neu-N mice, which are transgenic for rat neu and therefore express a tissue-specific tumor Ag (24, 26–29, 44). We have previously shown that these mice are tolerant to their transgene as compared with the FVB/N strain from which they were derived and that this tolerance is found both in the humoral and T cell arms of the immune response (24). However, we do not yet know the mechanism of this tolerance. It appears from our studies that neu-N mice given a whole-cell 3T3neuGM vaccine do not develop T cells specific for the immunodominant peptide in neu, although mice vaccinated with the heteroclitic version of the peptide show a degree of protection from neu-expressing tumor challenge. This may indicate that, although the predominant response to neu in neu-N mice is to other, less immunogenic epitopes, T cells specific for the immunodominant peptide may be induced. However, neu-N mouse-derived T cells may recognize the immunodominant epitope with a lower avidity as compared with FVB/N-derived T cells. This tolerance may occur in the thymus, in the periphery, or in both compartments. The identification of $RNEU_{420-429}$ as the immunodominant epitope contained in the rat neu protein will facilitate the further characterization of vaccine-induced immune responses in the rat neu-transgenic mouse model. To that end, an MHC/peptide tetramer can be made to identify T cells specific for the immunodominant epitope, because it is known that the restricting MHC molecule is $H-2D^q$. Identification of other epitopes in rat neu is also ongoing.

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